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Integrins act as signal transducers that regulate cell adhesion, spreading and motility on the extracellular matrix. Small GTP-binding proteins become activated in response to integrin ligation. Although members of the Rho family of small GTP-binding proteins control organization of the actin cytoskeleton, little is known about how they impact integrin-dependent cellular events on the ECM. Evidence is provided that cells transfected with Tiam1 (T lymphoma invasion and metastasis), a Rac activator, showed a 3-4 fold enhanced cell motility on collagen I, while cells expressing dominant negative Rac T17N failed to migrate in response to Tiam1. Overexpression of dominant positive small GTP-binding protein Rac did not significantly alter cell motility on collagen alone, however, its expression synergized with Raf kinase to activate MAP kinase and promote cell motility. Treatment of cells with a MAP kinase kinase (MEK) inhibitor, PD98059, and wortamanin, an inhibitor of phosphatidylinositide-3-kinase (PI3K), completely blocked Tiam1-induced cell motility. These results delineate roles of MAP kinase and PI3K in the regulation of integrin-mediated cell motility by the small GTP-binding protein Rac.

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INTRODUCTION

Metastasis of breast cancers contributes heavily to the severity and mortality of this disease (Marx J, 1993). There is substantial evidence that integrins contribute to the metastatic properties of breast cancer cells enabling them to invade and migrate to distant sites throughout the body (Brooks et al., 1994; Klemke et al., 1994). The integrins are a family of heterodimeric transmembrane proteins that bind to extracellular matrix proteins. The integrin-mediated binding regulates cell adhesion, spreading, motility, and cytoskeleton reorganization. However it is also apparent that integrin ligation also leads to activation of a range of signal transduction events (Schwartz et al., 1995). While the cytoplasmic domain of integrin α and β subunits do not have any intrinsic enzymatic activity, it appears they are able to couple with actin-binding proteins including several kinases (Parsons, 1996; Schwartz et al., 1995). Actin-binding proteins that colocalize with integrin in focal contact likely impact actin filament structure, the assemblies of these structure proteins are thought to play an important role in stabilizing cell adhesion and regulating cell shape, and motility.

Ras and related GTPases are GTP-binding proteins which regulate cell function via conversion between a GTP-bound active state and a GDP-bound inactive form. Considerable evidence has implicated Ras proteins as essential components of cellular events that regulate normal and abnormal cell growth and proliferation (Bokoch et al., 1993). The Rho family proteins are also members of the Ras superfamily of GTP-binding proteins. It includes Rho, Rac, and Cdc 42. It is clear that these proteins mediate morphological and cytoskeletal changes of tumor cells in Ras-inducing cell transformation (Qiu et al., 1995a and 1995b). Activation of Rac leads to the formation of lamillipodia and membrane ruffling, and Rac was implicated in cell movement and metastasis (Ridley et al., 1992). Rac has been also shown to have a direct role in the control of cell proliferation and the activation of Jun N-terminal kinase (JNK) signalling pathway (Coso et al., 1995; Minden et al., 1995).

This study provides evidence that small GTPase Rac is directly involved in integrin-mediated cell motility.

MATERIALS AND METHODS

Cells and Cell Culture

COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) with 10 % fetal bovine serum (FBS) and 50 μg/ml gentamycin. Prior to testing all cells were starved for 24 hours by replacing serum containing culture media with FBS-free media.

Antibodies and Reagents

Rabbit polyclonal antibodies to ERK2 was purchased form Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PD98059 is a drug that specifically inhibits MEK kinase and was graciously provided by Dr. Alan Saltiel (Park-Davis, Ann Arbor, MI). Myelin basic protein (MBP) was from Sigma. Collagen type 1 was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Fibronectin was from GIBCO. [γ-^{32}P]-ATP was purchased from Dupont-New England Nuclear.

Transfection of COS-7 Cells

COS-7 cells were transfected with lipofectamine (GIBCO) as described by the manufacturer. For each 10 cm plate, 20 μl of lipofectamine and 5 μg total DNA containing 0.5 μg of a reporter construct encoding ß-galactosidase (pCMV5-ß-galactosidase) were used. After 36 hour transfection, cells were starved in serum-free media for 24 hours before they were tested. Cells co-transfected with ß-galactosidase were developed using X-gal as a substrate. In some cases, cells were treated with the MEK inhibitor (PD98059, 25 μM) or Wortamanin (100 nM) for 20 minutes before cell migration assays.

Cell Migration Assay

Cell migration assays were performed using modified Boyden chambers containing polycarbonate membranes (tissue culture treated, 6.5 mm diameter, 10 μm thickness, 8 μm pores,

Transwell®; Costar, Cambridge, MA) as described previously (Klemke et al., 1994). Cells treated with the MEK kinase inhibitor PD98059 were allowed to migrate in the presence of drug for 6 hours. Migratory cells on the under surface were fixed and stained for expression of ß-galactosidase using X-gal as substrate. The number of blue cells was counted with an inverted microscope. Nonspecific or background migration was evaluated on BSA coated membranes and subtracted from all data points.

***In Vitro* Kinase Assay**

The ability of ERK to phosphorylate MBP was assayed according to Boulton et al. (1991). Briefly, 500 µg of protein from total cell lysates were precleared with protein A-Sepharose for 4 hours in the cold and then incubated with protein A-Sepharose coupled with anti-ERK antibodies (4 µg/100 µl stock bead suspension, Pierce) overnight in the cold. Immunoprcipitates were rinsed 3 times with RIPA and once with 0.1 M NaCl and 50 mM Hepes, pH 8.0 before incubation with 100 µl of reaction mixture containing 0.5 µCi [γ-^{32}P]ATP, 10 mM $MgCl_2$, 50 µM ATP, 1 mM dithiothreitol, 1 mM benzamidine, 0.3 mg/ml MBP, and 25 mM Hepes, pH 8.0, for 15 minutes at 30 ^{0}C. The reaction was stopped by adding a 100 µl of boiling SDS sample buffer before 8 µg of MBP was run on a 15 % polyacrylamide gel. The gel was then stained with coomassie blue, dried and exposed to imaging film overnight.

RESULTS

Expression of Tiam1 induces cell migration

Cell adhesion and migration on the ECM is mediated by integrin ligation and associated signal transduction event (Filardo et al., 1995; Klemke et al., 1994 and 1997). Protein phosphorylation is one of the earliest event detected in response to integrin stimulation. Tyrosine kinases, such as Src and focal adhesion kinase, and MAP kinase, are activated upon integrin

stimulation. The association of Sos, a guanine nucleotide exchange protein for Ras, with focal adhesion kinase, provides a mechanism by which integrins can regulate the Ras/MAK kinase pathway (Schlaepfer et al., 1994). Small GTP-binding protein Rac is required for Ras transformation, therefore, it is likely that Rac acts as a downstream effector of integrin in regulating the actin changes associated with cell adhesion.

Breast tumor cells like all transformed cells tend to be anchorage independent and thus demonstrate constitutively activated signaling molecules. Therefore, to begin to understand which of these signaling molecules might contribute to the metastatic capacity of breast cancer, I examined a non-malignant cell lines that could be induced to migrate by expression of activated signaling molecules. For this reason, COS-7 cells have been used as a model cell line to address this issue. To establish a potential role for Rac in integrin-mediated cell migration, COS-7 cells were transfected with Tiam1, a Rac activator,, and then allowed to migrate on a collagen substrate. Tiam1 was originally identified as an invasion-inducing gene from murine T lymphoma cells, it can induce metastatic activity in normally non-invasive cell lines (Habets et al., 1994). Its encoded protein shares striking homology with a number of guanine nucleotide exchange factors that are active on Rac/Rho proteins. As shown in Figure 1, Tiam1-expressing cells have a 3-4 fold increase in cell motility on collagen. Cotransfection with a dominant negative Rac T17N blocked Tiam1 enhancement of cell motility about 70%. These results suggest that Tiam1 can promote cell migration on collagen in a Rac-dependent manner.

Rac activation is necessary for integrin-mediated cell migration

The Rho-family of Ras-related GTPases is required for assembly of the actin cytoskeleton and associated focal complexes (Nobes et al., 1995). Rac stimulates the formation of membrane ruffling through actin cytoskeleton reorganization. To further investigate the role of Rac in integrin-mediated cell motility, dominant active and negative Rac was tested. As shown in Figure 2, dominant negative Rac and Cdc 42 blocked integrin-mediated cell migration significantly. Unexpectedly, overexpression of dominant active Rac Q61L did not alter cell migration on

collagen. These finding suggest that Rac is necessary but not sufficient for cell migration on collagen.

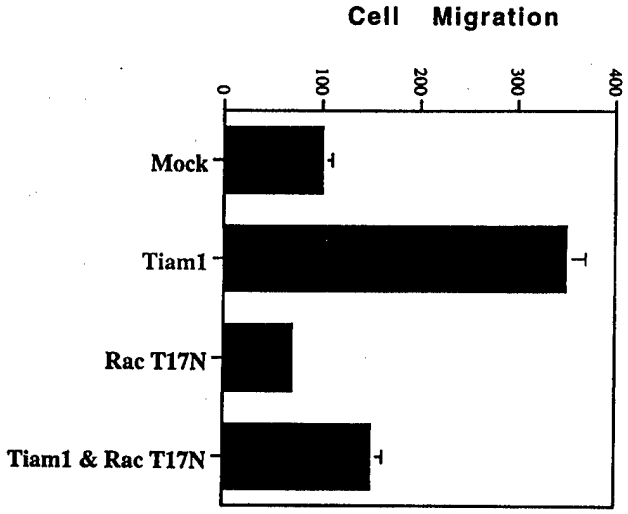

Figure 1. Induction of COS-7 cell migration following transfection with Tiam1

COS-7 cells were serum starved for 18 hours and allowed to migrate for 6 hours on collagen coated membranes after transient transfection with either the empty expression vector (control) or the expression vector containing Tiam1 or Rac T17N. In each case, cells were cotransfected with a ß-galactosidase containing vector for *in situ* ß galactosidase staining. This enabled us to enumerate only those migratory cells (ß-galactosidase positive) which had been transfected. In each case, transfection efficiency was the same. ß-galactosidase positive cells were enumerated by counting cells on the underside of the migration chamber using an Olympus inverted microscope.

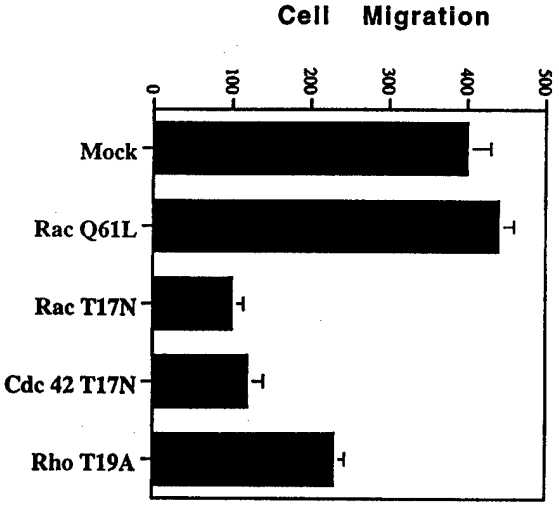

Figure 2. Effects of dominant negative Rac on integrin-mediated cell motility.

Dominant negative Rac T17N, Cdc42 T17N, Rho T19A or dominant positive Rac Q61L were transiently coexpressed with ß galactosidase in COS-7 cells. Cell migration assays were performed as previously described.

Rac and Raf synergize to activate MAP kinase and promote cell motility

Integrin ligation leads to the activation of focal adhesion kinase with subsequent activation of Ras/MAP kinase pathway (Schwartz et al. 1995). Recently, MAP kinase was shown to affect cell motility by regulating the activity of myosin light chain kinase (MLCK) (Klemke et al., 1997). Although Rac activates the JNK pathway and has little effect on MAP kinase activity, it appears to play an essential role in Ras transformation (Qiu et al., 1995a). Thus, it is possible that Rac is involved in the regulation of cell motility by MAP kinase. To address this issue, we examined the effects of Rac activation on MAP kinase activation and the induction of cell motility by Raf kinase. As shown in Figure 3, Rac expression together with low level Raf can not only activate MAP kinase, but can also promote cell migration on collagen.

To further study the mechanism by which Rac affects integrin-mediated cell motility, various inhibitors of potential downstream targets were tested. Pretreatment of cells with a MAP kinase kinase (MEK) inhibitor (PD98059), which specifically prevents its ability to promote both threonine and tyrosine phosphorylation of MAP kinase, completely blocks Tiam1-induced cell motility. This suggest MAP kinase is downstream of Rac and necessary for this migration event.

Recently, it was shown that Ras leads to activation of phosphatidylinositide-3-kinase (PI3K), Ras transformation and induction of membrane ruffling was blocked by inhibition of PI3K activity (Rodriguez-viciana et al., 1997). Interestingly, it has been suggested that Rac and PI3K lie in the same pathway, it is not clear which one of the signaling molecules lies upstream on the pathway. To test the effect of the activity of PI3K on Tiam1-induced motility, we used wortamanin, an inhibitor of PI3K. Cells were pretreated with this drug and allowed to migrate on a collagen substrate. As shown in Figure 4 that wortamanin blocked the cell migration. Together, these results imply roles of PI3K and MAK kinase in the regulation of integrin-mediated cell motility by Rac.

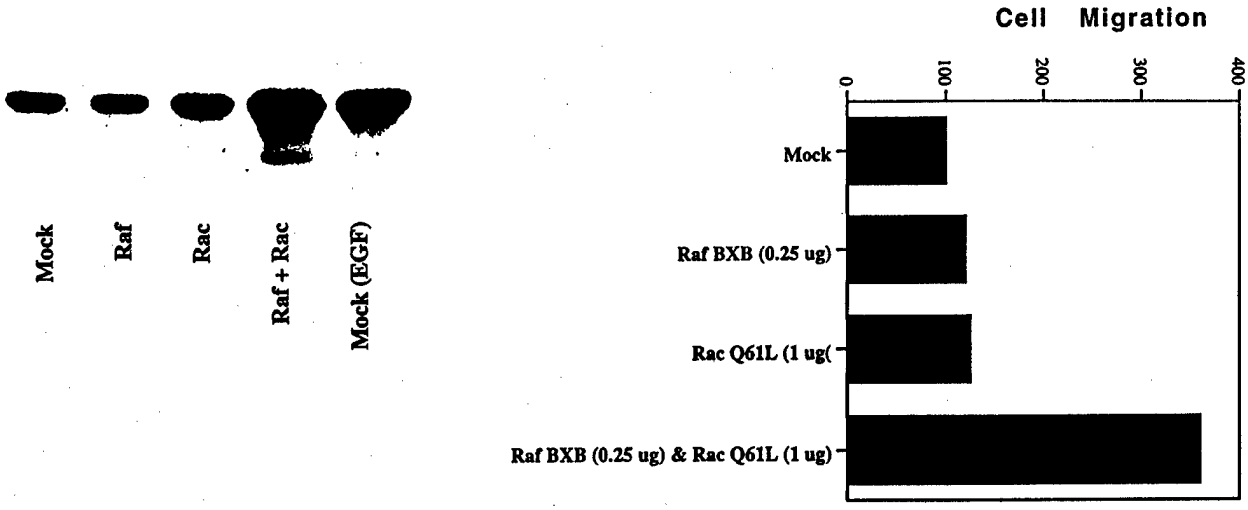

Figure 3. Rac synergizes with Raf to activate MAP kinase and promote cell migration. COS-7 cells were transfected with 0.25 ug of Raf BXB or 1 ug of Rac Q61L or both Raf BXB and Rac Q61L. A: In vitro MAP kinase assays. As a positive control, Mock transfected cells were treated with EGF (100 ng/ml) for 10 min before they were lysed. B: Cell migration assays.

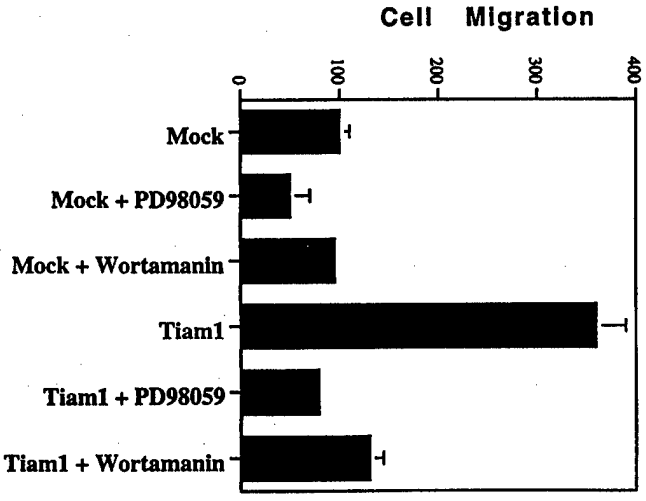

Figure 4. Effects of inhibitors on Tiam1-induced cell migration
Tiam1 was transiently coexpressed with ß galactosidase in COS-7 cells. After cells were treated with the MEK inhibitor (PD98059, 25 μM) or Wortamanin (100 nM) for 20 minutes before cell migration assays. Cell migration assays were performed as previously described.

CONCLUSIONS

Cell migration and invasion on ECM plays a critical role in tumor metastasis, angiogenesis as well as wound repair. Integrin ligation results in the assembly of multi-molecular focal complexes associated with the actin cytoskeleton. These structures are believed to be involved in integrin-mediated signal transduction events. It has previously shown that Rac and Rho are required for the formation of these focal complexes (Nobes et al., 1995). While it is clear that redistribution of focal complexes is necessary for directional cell movement, little is known about the role of Rho family GTPase Rac, one of the major regulator of these focal complexes, on cell migration. We have now examined the relationship between integrin ligation and Rac using the COS-7 cell model. When serum-starved cells were plated on polylysine or collagen-coated dishes, cells placed on a collagen substrate started to spread and show membrane ruffling after 15 minutes. *In vitro* kinase assays showed p21 activated kinase (PAK), one of the Rac downstream targets, was activated upon cell adhesion on the extracellular matrix (data not shown). These results suggest to us that integrin ligation may activates Rac which in turn may promote cell migration.

In this report, we provide evidence that small GTPase Rac is involved in the regulation of integrin-mediated cell migration. First, activation of endogenous Rac by a guanine nucleotide exchange factor, Tiam1, resulted in the increase of cell migration on collagen, and this increase can be blocked by coexpression of dominant negative Rac. Second, dominant negative Rac and Cdc 42 , when expressed in COS-7 cells, significantly decreased cell motility on collagen.

Overexpression of an active form of Rac did not result in any significant cell migration change, suggesting perhaps that the localization of Rac is also critical for directional cell movement. In fact, we observed that cells transfected with dominant positive Rac Q61L showed extensive cortical membrane ruffling, unlike Tiam1-transfected cells which only show membrane ruffles at their moving edges (data not shown).

The Ras/MAP kinase pathway has been recently shown involved in the regulation of cell migration through controlling the activity of MLCK (Klemke et al., 1997). We show here that MEK inhibitor blocks Tiam1-induced cell migration. Therefore, it is possible that Rac controls cell movement by impacting the activation of MAP kinase by integrin ligation. Recently, Rho has been shown to play a role in activation of MAP kinase by adhesion on fibronectin in NIH 3T3 cells (Renshaw et al., 1996). PI3K interacts with Ras.GTP but not Ras.GDP and is activated both in vitro and in vivo as a result of this interaction (Rodriguez-Viciana et al., 1994). PI3K has been implicated in the regulation of the actin cytoskeleton by Rac and growth factors such as PDGF and insulin (Wennstrom et al., 1994). As expected, we found inhibition of PI3K blocked cell motility induced by Tiam1.

In conclusion, we found Rac activity is required for integrin-mediated cell movement. Considering Rac signaling appears to be abnormal in breast cancer, this study will increase our understanding of abnormal cellular regulation in breast cancer and may lead to identification of direct therapeutic targets capable of inhibiting of tumor progression and metastasis

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REFERENCES

Bokoch G. M., Der C. J. 1993. Emerging concepts in the Ras superfamily of GTP-binding proteins. *FASEB J.* 7, 750-759.

Boulton T. G., and Cobb M. H. 1991. Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies. *Cell Regul.* 2, 357-371.

Brooks. P.C., Clark R.A. F., and Cheresh D.A.. 1994. Requirement of Integrin avb3 for angiogenesis. *Science*. 264, 569-571.

Coso O.A., Chiariello M.,Yu J., Teramoto H., Crespo P., Xu N., Miki T., and Gutkind J.S.. 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell*. 81, 1137-1146.

Dudley, D.T., Pang L., Decker S.J., Bridges A.J., and Saltiel A.R.. 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade.*Proc. Natl. Acad. Sci.* 92, 7686-7689.

Filardo, E.J., Brooks P.C., Deming S.L., Damsky C., and Cheresh D.A.. 1995. Requirement of the NPXY motif in the integrin b3 subunit cytoplasmic tail for melanoma cell migration in vitro and in vitro. *J. Cell Biol.* 130, 441-450.

Habets, G. G. M., Scholtes, E. H. M., Zuydgeest, D., van der Kammen, R. A., Stam, J. C., Berns, A., and Collard, J. G.. 1994. Identification of an invasion-inducing gene, *Tiam-1*, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. *Cell* 77, 537-549

Klemke R. L., Cai S., Giannini A. L., Gallagher P. J., and Cheresh D. A.. 1997. Regulation of cell motility by mitogen-activated protein kinase. *J. Cell Biol.* 137, 481-492.

Minden A., Lin A., Claret F., Abo A., and Karin M.. 1995. Selective activation of the JNK signaling cascade and C-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* 81, 1147-1157.

Nobes C. D. and Hall A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with stress fibers, lamellipodia, and filopodia. 81, 53-62.

Marx J.. 1993. Cellular changes on the route to metastasis. *Science* 259, 262-268.

Parsons T.H.. 1996. Integrin-mediated signaling: regulation by protein tyrosine kinases and small GTP-binding proteins. *Curr. Opin. Cell Biol.* 8, 146-152.

Qiu R. G., Chen J., Kirn D, McCormick F. and Symons M. 1995a. An essential role for Rac in

Ras transformation. *Science* 374, 457-459.

Qiu R. G., Chen J., McCormick F. and Symons M. 1995b. A role for Rho in Ras transformation. *Proc. Natl. Acad. Sci.* 92, 457-459.

Renshaw M. W., Toksoz D., and Schwartz M. A.. 1996. Invovlment of the small GTPase Rho in integrin-mediated activation of mitogen-activated protein kinase. *J. Biol. Chem.* 271, 21691-21694.

Ridley A. J., Paterson H. F., Johnston C. L., Diekmann D., and Hall, A.. 1992. The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling. *Cell* 70, 401-410.

Rodriguez-Viciana P.,Waterfield M. D., and Downward J. 1994. Phosphoinositide 3-OH kinase as a direct target of Ras. *Nature* 370, 527-532.

Rodriguez-Viciana P., Marte B., Pappin D., Das P., Waterfield M. D., Ridley A., and Downward J. 1997. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* 89, 11781-11785.

Schlaepfer, D.D., Hanks S.K., Hunter T., and van der Geer P.. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* 372:786-791.

Schwartz, M.A., Schaller M.D., Ginsberg M.H.. 1995. Integrins: Emerging paradigms of signal transduction. *Ann. Rev. Cell Dev. Biol.* 11, 549-599.

Wennstrom S., and Stephens L.. 1994. Activation of phosphoinositide-3-kinase is required for PDGF-stimulated membrane ruffling. *Curr. Biol* 4, 385-393